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Construction and expression of synthetic wild-type and mutant genes encoding porcine pancreatic colipase: tryptophan fluorescence studies

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Based on the known (95-residue) amino acid (aa) sequence of porcine pancreatic colipase (CLP), a cofactor of pancreatic lipase, a 297 bp gene was designed and assembled from eight synthetic, overlapping DNA fragments. Optimized for expression in bacteria, the CLP-encoding gene (*CLP*) was inserted into the *lacZ* gene fragment contained in the small expression vector, pUC8, and cloned in *Escherichia coli* JM109. Expression of this construct yielded a protein approx. 11 kDa in size, equivalent to CLP, with an M_r of 10 336, plus ten additional amino acids at the N-terminus. The recombinant CLP (reCLP) was solubilized from bacterial inclusion bodies and then purified and refolded. A mutant CLP gene, changing Tyr-55 to Trp, was then constructed by site-directed mutagenesis. Since porcine CLP contains no Trp, this strategy provided a protein with an internal fluorescent probe for biophysical studies. The presence of Trp in the mutant protein was confirmed using fluorescence spectroscopy. Both wild-type (wt) and mutant reCLP reacted on Western blots with an affinity-purified rabbit anti-CLP antibody, raised against native CLP. The Tyr-55 to Trp exchange did not affect the activity of reCLP. Fluorescence studies of the interaction between reCLP and the bile salt, taurodeoxycholate (TDOC), showed that Trp-55 in the hydrophobic binding site of mutant reCLP inserted into the interior of the bile salt micelle.

Abbreviations: aa, amino acid(s); Ap, ampicillin; β Gal, β -galactosidase; bp, base pair(s); cDNA, DNA complementary to RNA; CLP, colipase; *CLP*, colipase-encoding gene; CMC, critical micellar concentration; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; GuHCl, guanidine hydrochloride; IPTG, isopropyl- β -D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; λ_{max} , maximum fluorescence emission wavelength; LB, Luria-Bertani (medium); NP-40, Nonidet P-40; nt, nucleotide(s); NTSB, 2-nitro-5-(sulfothio)benzoate; oligo, oligodeoxynucleotide; ori, origin of DNA replication; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; po, promoter-operator; R, resistance/resistant; re, recombinant; SDS, sodium dodecyl sulfate; TDOC, sodium taurodeoxycholate; TE buffer, 0.1 M Tris-HCl (pH 8.2)/3 mM EDTA; wt, wild-type; XGal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

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Introduction

The pancreatic lipase-CLP enzyme system has been used as a model for studying interface catalysis, in which the enzyme, lipase, binds to the insoluble, hydrophobic substrate while remaining in its active conformation at the lipid/water interface. The enzymatic activity of lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is the hydrolysis of dietary triacylglycerols to free fatty acids and monoacylglycerols in the small intestine. CLP, a small, heat-stable protein cofactor containing five disulfide bridges (for reviews, see Refs. 28, 1, 3), functions in anchoring lipase to the bile-salt-covered surface of the lipid micelle [4,33]. Lipase by itself has a very low basal level of activity, which is enhanced by CLP approx. 10-fold.

During fat digestion, CLP binds to both lipase and

the bile salt micelle containing the triacylglycerol substrate; this micelle-binding region is believed to be a single, unique site on the CLP molecule [7,8]. Various physical studies have implicated certain Tyr residues, located within a well conserved hydrophobic domain, as being involved in micelle binding [26,12,9,36]. Spectroscopic analyses of dansylated porcine CLP have indicated that one of these residues, Tyr-55, most likely inserts into the hydrophobic interior of the bile salt micelle [21]. The aim of the present study was to approach this question using site-directed mutagenesis of a cloned, synthetic CLP gene, thereby substituting a Trp for Tyr-55 in the protein product. This conservative substitution allowed the use of intrinsic fluorescence spectroscopy in studying the mutant CLP, as opposed to chemical modification of the native protein.

Results and Discussion

Design, cloning and site-directed mutagenesis of the synthetic CLP gene

The CLP gene was assembled from 16 oligodeoxyribonucleotides (oligos) ranging from 31 to 42 bases in length (Fig. 1); the gene was cloned in halves, which were then ligated in vitro. The resulting construct was called pPCoL (Fig. 2). DNA sequencing of pPCoL by the dideoxynucleotide chain termination method of Sanger et al. [25] confirmed the absence of mutations in the cloned CLP gene.

Mutagenesis of Tyr-55 to Trp-55 was performed using the gapped duplex method [23] (Fig. 2). Colonies were screened by colony hybridization [30,17,35] using a synthetic oligo (16mer) probe (Fig. 4). Plasmid DNA from one positive clone, i.e., containing both the wild-type (wt) plasmid, pPCoL, and the mutant plasmid, called pPCoL-Trp-55, was used to retransform JM109 cells. Desired mutant clones were selected on the basis of loss of an *Rsa*I restriction site located at the Tyr-55 codon in the CLP gene (see Fig. 1). The two base changes were confirmed by dideoxy sequencing of plasmid DNA from one of these clones; the rest of the gene sequence was found to be free of mutation.

Expression of the synthetic CLP genes

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of lysates from IPTG-induced JM109 clones harboring pPCoL or pPCoL-Trp-55 revealed the presence of stable protein products with an M_r of approx. 11 000, i.e., equivalent to proCLP with ten additional amino acids at the N-terminus (Fig. 3A, lanes 4 and 5). These additional amino acids most likely protected the reCLP from rapid degradation by *E. coli* proteinases. Densitometric scanning of the Coomassie blue-stained gel indicated that the wt and mutant reCLPs were present in significant amounts, i.e., approx. 1–2% of the total cell

protein. These proteins were recognized by the anti-CLP antibody on a Western blot (Fig. 3B, lanes 4 and 5), and therefore contained epitopes of native CLP. No CLP was detected in uninduced cells (Fig. 3A and 3B, lanes 3).

High speed centrifugation of the cell lysates in a Beckman Airfuge, followed by SDS-PAGE/Western blot analysis of the supernate and pellet fractions, revealed that the reCLP was localized entirely in the insoluble fraction, most likely in the form of dense aggregates known as inclusion bodies.

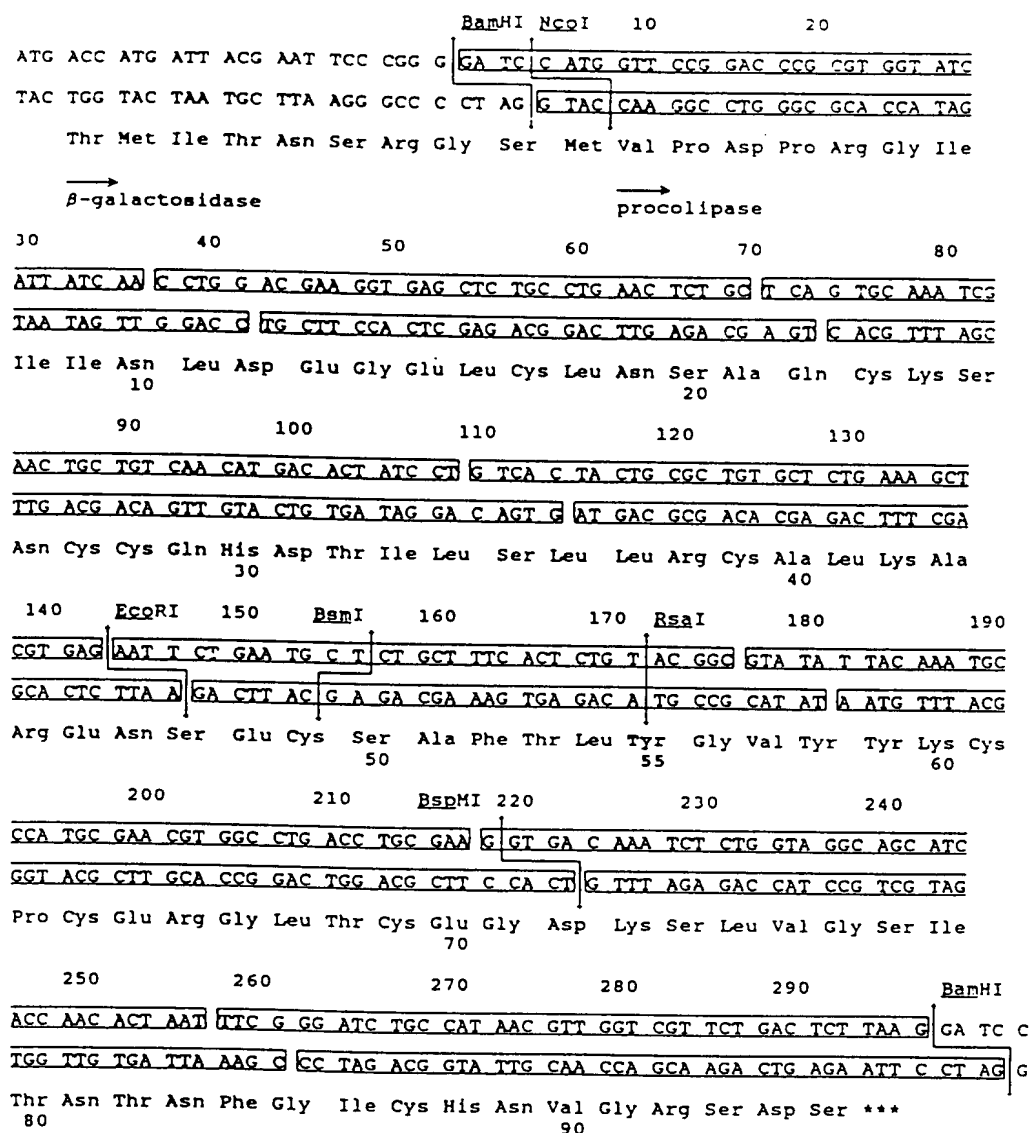
Bacterial cell lysis and isolation of inclusion bodies

A method was developed based on published procedures [18,31,22] to purify reCLP from the insoluble fraction of lysates from induced bacterial cells (Fig. 5). Since the inclusion bodies sedimented rapidly during low speed centrifugation, most of the bacterial proteins remained in the supernate. The remaining bacterial membrane proteins, which associated tightly with the inclusion bodies (for review, see Ref. 19), were largely removed by washing the pellet in the Triton X-100/EDTA solution (Fig. 5). At this stage, the CLP was already more than 90% pure, as estimated from SDS-PAGE (see Fig. 5, lane 2). The amount of CLP present in the inclusions pellet was, on the average, approx. 5–10 mg per l of culture.

Purification and refolding of reCLP

Generally speaking, when a recombinant protein is purified from bacterial inclusion bodies it must be, first of all, solubilized using a denaturant, and secondly, refolded into its native conformation using a combination of conditions specific for that protein. The recovery of biological activity, as in the case of an enzyme or cofactor, however, rarely exceeds 50–60% of the native activity. For reCLP, inclusion bodies were solubilized using 6 M guanidine hydrochloride (GuHCl), together with sodium sulfite which cleaves any disulfide bridges, either intramolecular or intermolecular, present in the inclusion bodies or formed during solubilization. This 'partial sulfonation' procedure yielded the highest percent of refolding of the native cofactor (see below) of several procedures attempted, including 'full sulfonation' using 2-nitro-5-(sulfothio)benzoate (NTSB) [31], and the use of dithiothreitol (DTT) in place of Na_2SO_3 .

After solubilization, the reCLP was desalted using Sephadex G-25 chromatography rather than dialysis vs. 0.1 M Tris-HCl (pH 7.4 or 8.0) which resulted in precipitation of the protein. The reCLP could be maintained in soluble form by elution from the G-25 column in 0.1 M acetic acid (Fig. 5, lane 2). Most likely, the dilution achieved on the Sephadex column was sufficient to prevent aggregation of the protein as the denaturant was being removed. Also, some material present in the inclusion bodies, which could contribute



Nucleotide (nt) sequence and design of the synthetic porcine CLP gene. The 297 bp DNA sequence was deduced from the aa sequence (residues) [29] of the proform of porcine CLP [2,24]. The CLP gene is shown inserted in the *Bam*HI site of pUC8, which contains the *lac* operon and *lacZ'*, coding for the β-galactosidase (βGal) α-peptide [34]. The 16 oligos, synthesized using β-cyanoethylphosphoramidite chemistry on the Pharmacia Gene Assembler, are indicated as boxed sequences. Aa are numbered starting with the N-terminal valine of proCLP; shown in boldface. Nt numbering begins with the first base of the synthetic gene sequence. The nt sequence was optimized for preferred usage in bacteria according to the protocol of Grantham et al. [14]. The synthetic CLP gene product contains ten additional aa at the N-terminus which are derived from βGal (first 6) and multiple cloning site codons in pUC8. Some unique restriction sites used in cloning and site-directed mutagenesis are labeled.

protein aggregation [11], was possibly removed by column.

The desalted crude reCLP in acetic acid was concentrated and then purified on a Sephadex G-50 column in the presence of 2-mercaptoethanol, to prevent formation of intermolecular and/or incorrect disulfide bridges during chromatography. G-50 fractions containing Trp-55-CLP with the highest purity as determined by SDS-PAGE were pooled (Fig. 5, lane 4)

and used for fluorescence studies (see below). The absence of oligomers cross-linked by disulfide bridges in the purified reCLP sample was confirmed by non-reducing SDS-PAGE (Fig. 5, lane 5).

For activity assay, aliquots of the Sephadex G-50 pool were lyophilized and the reCLP refolded by dissolving the protein in 0.1 M Tris-HCl (pH 8.2)/3 mM EDTA (TE buffer) [31] at room temperature for 24 h. Since not all of the protein was soluble in the buffer,

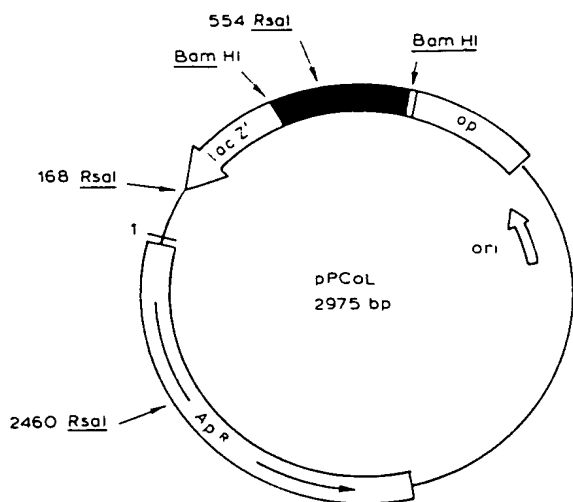


Fig. 2. Assembly of the synthetic CLP gene: construction of pPCoL. Plasmid is not drawn to scale. Complementary (phosphorylated) oligos were annealed in pairs and then ligated to form the gene halves, which were inserted in separate reactions into the *Bam*HI and *Eco*RI sites of pUC8. The resulting constructs were used to transform *E. coli* JM109, which are deficient in β Gal activity [37]. Desired colonies appeared white on agar plates containing the lac inducer, isopropyl- β -D-thiogalactopyranoside (IPTG), and indicator dye. 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (XGal), and were ampicillin (Ap)-resistant. Inserts were excised from their respective plasmids by restriction enzyme digestion, ligated (at the *Eco*RI site), and then inserted into the *Bam*HI site of pUC8; solid area represents the full-length CLP gene. Arrow indicates direction of transcription from the lac promoter-operator (po). For site-directed mutagenesis, pPCoL was cleaved at the unique *Sca*I site in the β -lactamase gene, and in a separate reaction, with *Bam*HI to remove the CLP gene insert. The resulting plasmid fragments were mixed with the mutagenic 69mer (unphosphorylated at the 5' end) (Fig. 4), denatured, and reannealed. Gaps in the DNA duplex were filled by use of the Klenow (large) fragment of *E. coli* DNA polymerase I, and the reaction mixture (unligated) was used to transform *E. coli* JM105 [37]. Ap^R ampicillin-resistance gene; ori, origin of DNA replication.

however, only the supernate, which contained from 0.1 to 1 mg/ml of protein, was assayed for CLP stimulation of pancreatic lipase activity using the tributyrin emulsion pH titration assay [6]. Apparently, concentra-

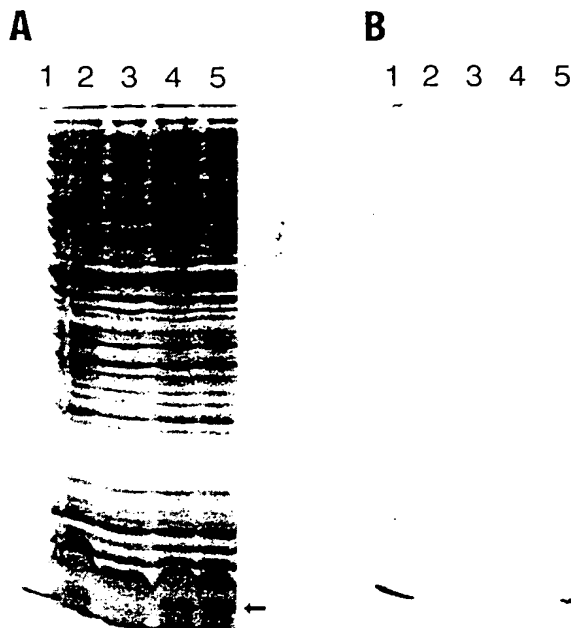


Fig. 3. Expression of the synthetic wt and mutant CLP genes. Bacteria were grown in Luria-Bertani (LB) medium containing Ap (100 μ g/ml) to $A_{600} = 0.7$, and induced with 1 mM IPTG or left uninduced, as indicated. Cells from 0.5 ml of culture were harvested after 3 h, washed in 50 mM Tris-HCl (pH 7.5), lysed in $2\times$ Laemmli sample buffer, boiled for 10 min, and the lysates loaded onto a 12.5% SDS-PA gel [15]; the gel was stained with Coomassie brilliant blue (A). Protein bands were blotted onto nitrocellulose [32] and probed with an affinity-purified rabbit anti-CLP antibody, followed by alkaline phosphatase-linked goat anti-rabbit antibody (Bio-Rad) (B). The rabbit antibody was raised against purified porcine pancreatic CLP (proform) [6], and then purified on a CLP-affinity column prepared using CNBr-activated Sepharose 4B (Pharmacia). Lane 1, porcine CLP standard, 2.5 μ g; lane 2, control cells (pUC8 without CLP gene); lane 3, cells with wt CLP gene (pPCoL), uninduced; lane 4, same as lane 3, except induced; and lane 5, cells with mutant CLP gene (pPCoL-Trp-55). Arrows indicate reCLP.

tion of the reCLP by lyophilization together with removal of the acetic acid resulted in disulfide bridge formation and protein aggregation in the Tris-HCl buffer; these aggregates could only be solubilized in

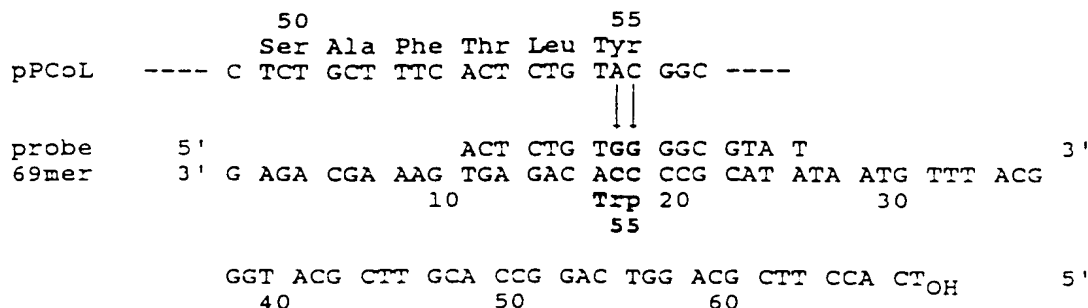


Fig. 4. Design of the synthetic oligos used in site-directed mutagenesis. Sequences of the synthetic 69mer (mutagenic) and 16mer (probe), which contain the codon for Tyr-55 (TAC) changed to that of Trp-55 (TGG), are shown (written in the 3' to 5' direction for the 69mer). For colony hybridization, the probe was radiolabeled at the 5' end with [γ - 32 P]ATP and T4 polynucleotide kinase.

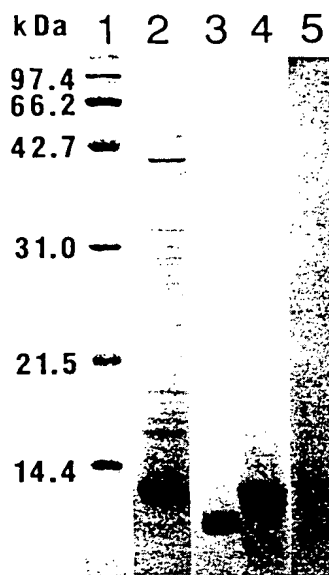


Fig. 5. SDS-PAGE analysis of Trp-55-CLP purification. IPTG-induced bacteria from 3 l of culture were lysed in 50 mM Tris-HCl (pH 7.5) containing 0.19 mg/ml lysozyme, 0.075% (v/v) Nonidet P-40 (NP-40), 9.8% (w/v) sucrose, 0.19 M NaCl and 10 mM EDTA for 30 min on ice [22], without phenylmethylsulfonyl fluoride (PMSF). After sonication for three times 20 s, the cell lysates were centrifuged at $12000 \times g$ for 10 min and the pellet was washed in 10–20 vol. of 50 mM Tris-HCl (pH 8.0)/0.5% (v/v) Triton X-100/50 mM NaCl/10 mM EDTA [18]. The washed inclusion bodies were then solubilized in 10 vol. of 6 M guanidine hydrochloride (GuHCl)/0.3 M Na_2SO_3 (pH 8.0) [31] and left overnight at room temperature to dissolve completely. 19 ml were then filtered using a 0.4 μm Nalgene Type A filter unit (polycarbonate membrane) and desalted on a 2.5×38 cm Sephadex G-25 column equilibrated with 0.1 M acetic acid. Fractions containing protein were pooled for purification on Sephadex G-50. A 0.8×24 cm G-50 column, equilibrated with 10% (v/v) acetic acid containing 2% (v/v) 2-mercaptoethanol, was loaded with 0.75 ml of the desalted and concentrated, crude Trp-55-CLP to which 2% (v/v) 2-mercaptoethanol had been added. The figure is a composite of two Coomassie blue-stained 12.5% PA gels. Lane 1, protein standards (Bio-Rad, Low range); lane 2, Sephadex G-25 pool (desalted crude Trp-55-CLP); lane 3, porcine CLP standard, 2.5 μg ; lane 4, Sephadex G-50 pool (purified Trp-55-CLP), 6.8 μg ; and lane 5, same as lane 4 except nonreduced, i.e., without 2-mercaptoethanol in the sample buffer.

the presence of 2-mercaptoethanol. Specific activity values were expressed as percent activity of a 0.0135 mg/ml CLP standard solution (set at 100%). The specific activities of both the purified Trp-55-CLP and wt reCLP were found to be 5–10%. A similar level of activity was recovered when native porcine CLP was denatured and refolded under identical conditions (E.G. Ernst, P.L. Hundley and W.D. Behnke, unpublished observations). Furthermore, the addition of oxidized and reduced glutathione (GSSG and GSH) in a 1:2, 4:1 or 10:1 ratio did not increase the final activity compared to buffer alone.

One explanation of why CLP does not refold completely once reduced and denatured is that the molecule

which is isolated from porcine pancreas most likely lacks additional amino acids at its N-terminus. Such a signal sequence, as was recently found for human CLP [16], as well as additional residues at the C-terminus [3] could be necessary for efficient refolding of both native and bacterially-produced CLP. Another factor to consider is the percentage of α -helix in CLP. Because these regions of secondary structure are postulated to act as nucleation centers for protein folding (for review, see Ref. 10), it follows that proteins with a high α -helical content could fold more efficiently than proteins which lack a large amount of ordered structure. Since CLP has very little – only 5% – α helix and a preponderance of β - and unordered structure [5], the formation of nucleation centers during refolding is probably very inefficient. The refolding process is further complicated by the large number of disulfide bridges – five – (for a M_r of only 10 000–11 000), which can form stable but incorrect folding intermediates.

As mentioned above, 100% activity is rarely obtained for enzymes and cofactors which are isolated from bacterial inclusion bodies. Attempts were made in our laboratory to produce CLP in soluble form in the periplasmic space of *E. coli* using various bacterial secretion vectors; however, these constructs were found to be toxic to the bacterial host cells (E.G. Ernst and W.D. Behnke, unpublished results). Thus, the only way to obtain reCLP in sufficient quantities for our purposes was to produce it in the form of stable inclusion bodies.

Fluorescence studies of Trp-55-CLP

The presence of a Trp residue in the mutant CLP gene product was confirmed by the fluorescence emission spectrum shown in Fig. 6 (solid curve). The maximum emission wavelength (λ_{max}) of 335 nm is characteristic for Trp. The spectrum of native porcine CLP, which lacks a Trp, has a λ_{max} at 303 nm due to the three Tyr residues (Fig. 6, dashed curve). The fluorescence emission spectrum of Trp-55-CLP resulting from excitation at 280 nm (Fig. 7A, solid curve) had a half-maximum spectral width of 58 nm. At an excitation wavelength of 295 nm, the emission spectrum was of lower intensity (dashed curve), with a spectral width of 55 nm and a relative quantum yield that was decreased by 53%. This difference between the two spectra can be explained by a fluorescence energy transfer from Tyr to Trp-55 upon excitation at 280 nm. The two maxima at 230 nm and 280 nm in the fluorescence excitation spectra (Fig. 7B) coincided for Trp-55- and native porcine CLP, as did the relative intensities of these bands. These data characterize the mutant protein as a porcine CLP. In contrast, equine CLP, containing a single Trp at position 52, has a different excitation spectrum [20].

In order to show directly that Tyr-55 inserts into the

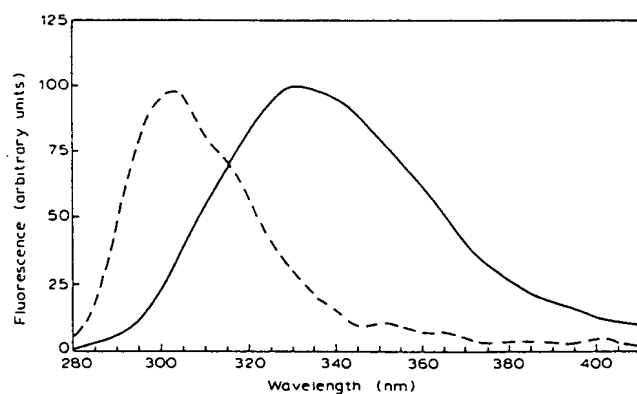


Fig. 6. Fluorescence emission spectra of native porcine and Trp-55-CLP. Native CLP was prepared from porcine pancreas using the method of Chapus et al. [6]; recombinant Trp-55-CLP was isolated from bacterial inclusion bodies. The protein concentrations of the native (dashed line) and mutant (solid line) CLPs were $5 \mu\text{M}$ and $10 \mu\text{M}$, respectively, in 0.1 M Tris-HCl (pH 7.5). Spectra were obtained at 25°C using a Perkin-Elmer Model MPF-44A fluorescence spectrophotometer equipped with a DCSU-2 corrected spectra unit. The excitation wavelength was 265 nm , and the baseline due to buffer was subtracted from the spectra.

bile salt micelle [21], the effect of increasing concentrations of the bile salt, taurodeoxycholate (TDOC), above the critical micellar concentration ($\text{CMC} = 1 \text{ mM}$) on the fluorescence emission spectrum of Trp-55-CLP was studied. Fig. 8A shows that addition of 10 mM TDOC causes a 53% increase in the fluorescence intensity, and a blue shift in the λ_{max} of 5 nm , to 330 nm (upper curves). This effect was maximal at 5 mM TDOC. In contrast, when the Trp side chain is completely free (i.e., not part of a folded protein structure), as in the model peptide, *N*-acetyl-L-tryptophanamide, neither the fluorescence intensity nor the λ_{max} of 355 nm were affected by addition of 5 mM TDOC (Fig. 8B, upper curves). Equine CLP, in which the single Trp at position 52 has been shown to interact with bile salt micelles, behaved similarly to Trp-55-CLP, showing a substantial increase and shift in its fluorescence emis-

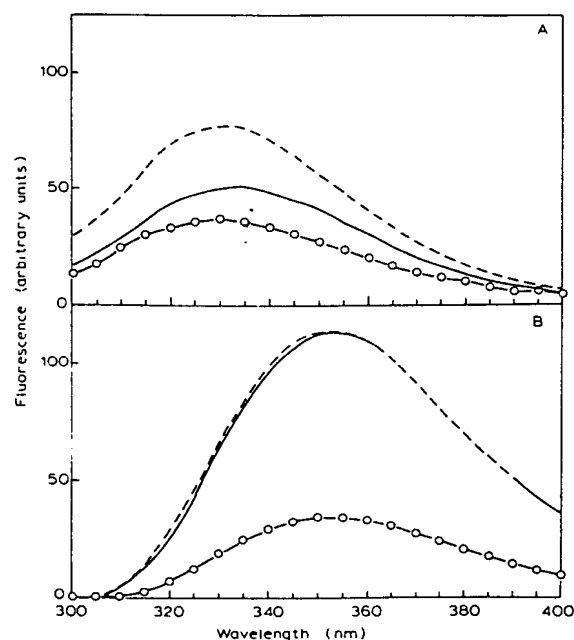


Fig. 8. Effects of TDOC and 2-mercaptoethanol on the fluorescence emission spectra of Trp-55-CLP and *N*-acetyl-L-tryptophanamide. The Trp-55-CLP sample (A) was prepared as in Fig. 7. The *N*-acetyl-L-tryptophanamide (panel B) concentration was $41 \mu\text{M}$ in 0.1 M Tris-HCl (pH 7.5). TDOC was added from a 0.5 M stock solution. The excitation wavelength was 280 nm , and the temperature was 25°C . Emission spectra are shown in the absence of TDOC (solid line); in the presence of 10 mM (A) or 5 mM (B) TDOC (dashed line); and in the presence of 25 mM TDOC plus 2% (v/v) 2-mercaptoethanol (solid line, open circles).

sion spectrum, at 4 mM TDOC [13]. The two CLP species thus have similar micelle-binding affinities. Fig. 9 shows the Scatchard analysis of the binding of TDOC micelles to Trp-55-CLP. The K_d value of $1.7 \cdot 10^{-4} \text{ M}$, computed from the fluorescence data agrees well with the value ($1.5 \cdot 10^{-4} \text{ M}$) reported for equine CLP [20]. Also, the number of micelle-binding sites ($n = 1.28$) confirms a 1:1 binding stoichiometry between Trp-55-

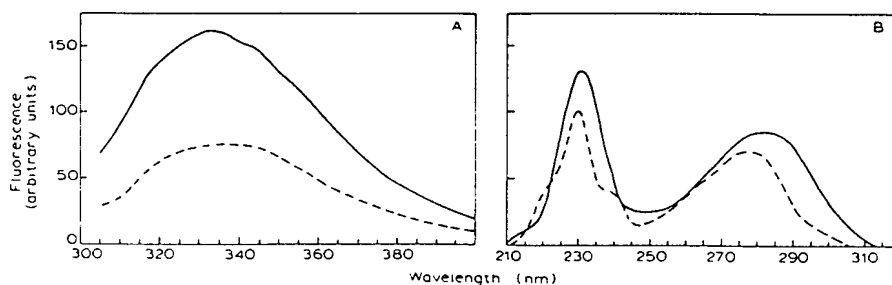


Fig. 7. The emission spectra of recombinant Trp-55-CLP (A) were obtained using fluorescence excitation wavelengths of 280 nm (solid line) and 295 nm (dashed line). To obtain the excitation spectra (B) of mutant (solid line) and native porcine CLP (dashed line), the emission wavelength was fixed at 335 nm . Sample preparation: the Sephadex G-50 pool containing purified Trp-55-CLP (Fig. 5) was dialyzed vs. 10% (v/v) acetic acid, which was then exchanged for 0.1 M Tris-HCl (pH 7.5) using a Pharmacia NAP-10 column. Native CLP was prepared as described in Fig. 6. The protein concentrations were $3 \mu\text{M}$, and the spectra were obtained at 25°C .

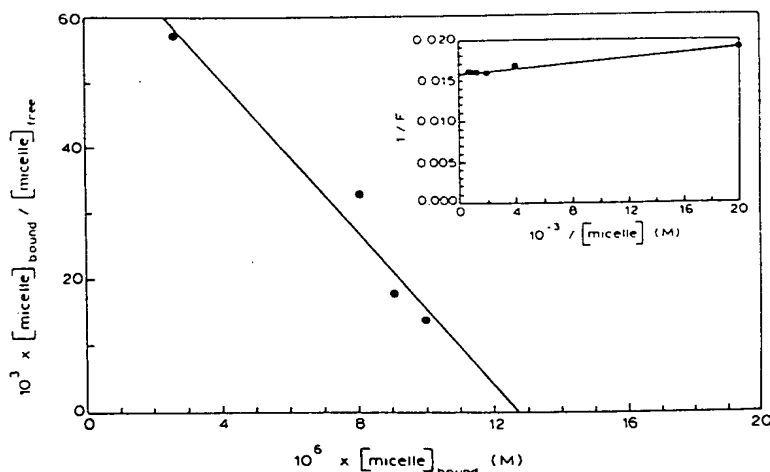


Fig. 9. Scatchard analysis [27] of TDOC micelle binding to Trp-55-CLP. Increasing concentrations of 1 mM (CMC), 5 mM, 10 mM and 15 mM TDOC were added to a 10 μ M solution of recombinant Trp-55-CLP in 0.01 M Tris-HCl (pH 7.5); fluorescence emission spectra were obtained as in Fig. 8, except using an excitation wavelength of 265 nm. Data taken at the λ_{max} values were used for the double-reciprocal plot of $1/\text{fluorescence}$ vs. $1/[\text{micelle}]$ (inset), based on 20 TDOC monomers per micelle [7,26]. The limiting fluorescence intensity was obtained by extrapolating to infinite $[\text{micelle}]$. The Scatchard plot was constructed assuming a single micelle-binding site per CLP molecule [7,8]. The K_d value was calculated from the slope, and the number of binding sites (n) was taken from the intercept (see Results).

CLP and the TDOC micelle, as predicted from studies on native CLP using other physical methods [7,8].

As a control, Trp-55-CLP was reduced with 2-mercaptoethanol, which normally abolishes native CLP activity and, thus, certain aspects of tertiary structure; this treatment resulted in a loss of the fluorescence enhancement due to TDOC (Fig. 8A, lower curve). This elimination of the TDOC effect was also observed for equine CLP (E.G. Ernst, unpublished observation) and can be explained by disruption of the interaction between CLP and the TDOC micelle. It is interesting that, in the presence of 2-mercaptoethanol, the blue shift in the λ_{max} was not eliminated; this phenomenon also occurred with equine CLP (E.G. Ernst, unpublished observation). Perhaps, when the tertiary structures of the proteins are lost, the Trp residues still remain partially buried, and, thus, protected by hydrophobic residues (residual secondary structure) in the polypeptide chain. In addition, the lower curve in Fig. 8A represents a 27% decrease in the baseline fluorescence emission of Trp-55, most likely due to partial quenching by the 2-mercaptoethanol. This effect was not as great as for *N*-acetyl-L-tryptophanamide, where the Trp is completely exposed to solvent, and the fluorescence was decreased by 70% (Fig. 8B, lower curve).

Conclusions

(1) The enhancement of fluorescence intensity and blue shift of the λ_{max} in the presence of TDOC results from an interaction of Trp-55 in mutant CLP with the bile salt micelle. This interaction occurs only when the

micelle-binding domain has the correct tertiary structure, and is abolished when the protein is denatured by the use of 2-mercaptoethanol.

(2) The fluorescence changes observed with Trp-55-CLP are far more extensive than would be expected by activity measurements (based on analogy to the equine system). Results of the Scatchard analysis indeed support the conclusion that an intact micelle-binding site is present (see above); the lipase-binding site, however, may be only partially folded.

(3) The fluorescence studies on Trp-55-CLP confirm the data generated by chemical modification of Tyr-55 (e.g., nitration, reduction, treatment with dansyl chloride) and their subsequent interpretations. Because an intrinsic natural fluorescent probe has been successfully introduced by site-directed mutagenesis, reservations concerning the attachment of large fluorescent molecules to CLP and any conformational perturbations this might cause are eliminated. Thus, the present study proves directly that Tyr-55 in porcine pancreatic CLP inserts into the hydrophobic interior of the bile salt micelle.

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